Biology of the Cell, **63** (1988) 361–366

Original article

Degradation of yolk in the brine shrimp *Artemia*. Biochemical and morphological studies on the involvement of the lysosomal system

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(Received 10-8-1987; accepted 20-4-1988)

The degradation of yolk granules during the development of *Artemia* was studied. The results obtained suggest that lysosomes are involved in the process. In homogenates of embryos and larvae at different stages of development, the distribution of 2 lysosomal markers, acid phosphatase and cathepsin B, was studied by sucrose isopycnic gradient centrifugation. Three peaks of enzyme activity of densities >1.3 and around 1.25 and 1.18 were observed. As revealed by electron microscope analysis, the 3 peaks were found to be associated with increasingly degraded yolk structures which stained for acid phosphatase. The process can be mimicked *in vitro* by incubating isolated yolk granules and lysosomes. The enzyme activity levels of the 3 peaks observed during development presented an oscillatory pattern, suggesting that degradation of yolk is cyclic. Five cycles of degradation were observed during the initial 60 hr of development.

yolk degradation — *Artemia* — lysosomal involvement — biochemical and morphological studies — cytochemical staining

INTRODUCTION

The first steps in the developmental program take place in the absence of nutrient supply. The components necessary for the intense synthetic processes come from the degradation of maternal reserve sources. However, neither the mechanism of degradation of stored components nor its regulation are well understood at present.

The most important storage structure found in the oocyte of many animal species is the yolk granule [2]. Yolk granules are rather unstable structures. We previously reported a method for the isolation of intact yolk granules from *Artemia* [18], using a medium that preserves the subcellular embryonic structures [9, 15]. The availability of this method has allowed us to study the process of yolk degradation in *Artemia*. The association of lysosomal enzyme activities with yolk granules has been observed in different systems by electron microscopy [12, 16, 17], but the possible involvement of lysosomes in the degradation of yolk has not been further investigated. We have demonstrated the occurrence of lysosomes and several acid hydrolytic activities in the dormant embryo of *Artemia*. The activity of these enzymes increases during development, at the time of maximum yolk degradation [13]. Among the lysosomal activities, we have found a cathepsin B-like proteinase which is able to degrade the yolk protein *in vitro* [13] with the specific pattern observed *in vivo* [5]. In this paper we report on the degradation of the yolk granules during the first 60 hr after the activation of the dormant gastrula, a period in which more than 80% of the yolk granules are metabolized. The biochemical and electron microscope observations suggest that the lysosomal system may be involved in the process of yolk degradation.

MATERIALS AND METHODS

Materials

Artemia cysts from San Francisco Bay (San Francisco Bay Brand, CA were used. Chemical were of analytical grade.

Culture, handing, counting, and homogenization of embryos and larvae

Dormant embryos (cysts) were subjected to complete dechorionization [18] before homogenization and/or incubation. This treatment improves synchrony and hatching. Culture was accomplished in a suitable saline medium and larvae were synchronized, collected, and counted as described previously [18]. Embryos and larvae at different stages of development were homogenized in 3 vol/g of a Ficoll medium [13]. This medium has proved to preserve the subcellular structures of *Artemia* [15, 18].

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Gradients

Two mol of homogenates from embryos and larvae at different stages of development were loaded on a sucrose isopynic gradient (0.96–2 M) containing 5 mM EDTA and 100 μg/ml of soybean trypsin inhibitor and the gradients, were run at 25,000 rev/min for 14 hr in an SW27 rotor. Lysosomal enzyme-containing structures were detected by assaying in the fractions of the gradient activity of 2 lysosomal markers, acid phosphatase and cathepsin B. Yolk granules sediment to the bottom of the gradient in these conditions [9, 18].

Assays

Lysosomal enzyme activities were assayed after release of latency as described by Perona & Vallejo [13]. Protein was determined by the method of Bradford [4].

Electron microscopy

Samples the fractions of the sucrose gradients were analyzed by electron microscopy. When samples had a high sucrose content, they were diluted to 0.3 M sucrose by adding distilled water, and centrifuged. The samples were then fixed in Karnovsky's glutaraldehyde-paraformaldehyde fixative [8]. Other details were as described [18].

To detect the presence of acid phosphatase, the samples were subsequently incubated in Gomori's medium in the presence and the absence of substrate.

RESULTS

Biochemical studies of lysosomal enzyme-containing structures during the development of Artemia

Lysosomal degradative processs start with the formation of the degradative structure, as a result of the fusion of lysosomes with the material to be degraded [6]. The resulting hetero- or autophagolysosomes may differ in buoyant density, depending on the nature and or integrity of the material to be degraded. Populations of these structures differing in buoyant density can be isolated by sucrose isopycnic gradient centrifugation [11]. Accordingly, study of autophagosomes in *Artemia* could give some information about the material that lysosomes were degrading during development.

Embryos and larvae at different stages of development were homogenized and the homogenates centrifuged through sucrose isopycnic gradients. Two lysosomal enzyme activities, acid phosphatase and cathepsin B, as well as protein content were determined in the fractions of the gradients. The results observed at 3 times of development are shown in Figure 1. The lysosomal enzymatic activities and protein presented a similar pattern of sedimentation. Most of the activity in the homogenates of dormant embryos sedimented to the bottom of the gradient (Fig. 1a). However, 3 populations of lysosomal enzyme-containing structures could be identified in larvae homogenates (Fig. 1b, c). The population of highest density sedimented at the bottom of the gradient (density > 1.3) and the other 2 populations sedimented at density 1.22–1.24 and 1.19–1.17, respectively. The density of the 2 heavier populations of phagolysosomes (> 1.3 and around 1.25, respectively) indicated that lysosomes were involved in the

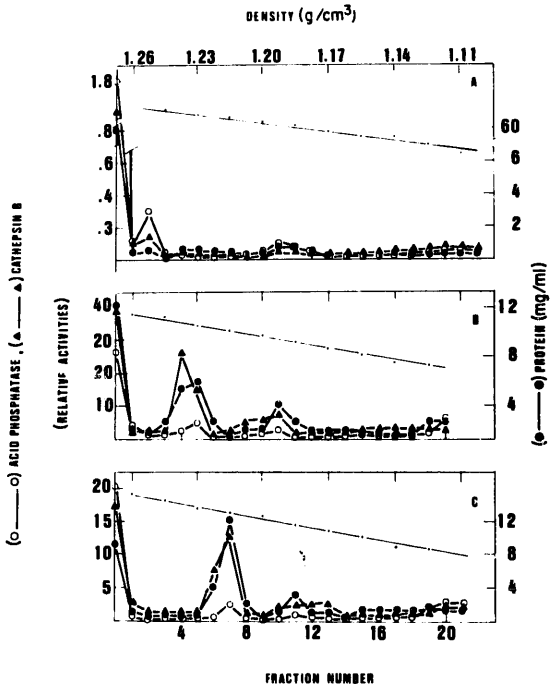

FIGURE 1. – Sedimentation by sucrose isopycnic gradient centrifugation of lysosomal enzyme markers during the early development of *Artemia*. Homogenates (2 ml) of dormant embryos (a) and larvae of total development of 24 hr (b) and 36 hr (c) were loaded on the sucrose isopynic gradient and centrifuged. Fractions of 0.5 ml were collected. Acid phosphatase, cathepsin B, and protein were determined in each fraction. The experiments were repeated 2 or 3 times, with no significant variation observed. The enzyme measurements corresponding to data plotted were at least 5-fold above the sensitivity limit of the corresponding method [7, 13].

degradation of material of high density. On the other hand, the similar pattern of sedimentation shown by lysosomal enzyme activities and the protein suggested that lysosomes were involved during *Artemia* development in massive degradation processes. In Figure 2 the morphology of the 3 different lysosomal enzyme-containing structures is shown, indicating that the massive degradation was related to yolk granules. Indeed, these organelles contain more than 80% of the protein of the animal [18].

Microscope observation of the structures of the gradient suggested that the structures of lighter density (Fig. 2c, d), presumably resulting from the degradation of the heavier (Fig. 2a, b), were rich in lysosomal enzyme activity. We also observed that the mixing of isolated yolk granules from dormant embryos with lysosomes from the same source gave rise to a peak of activity of density around 1.23 (Fig. 3). This *in vitro* result supported the notion that the degradation of yolk granules can be induced upon addition or incorporation of lysosomes. In the control gradient corresponding to yolk granules (Fig. 3b), the enzymatic activity and protein sedimented at the bottom of the gradient (density > 1.3). In the control correspon-

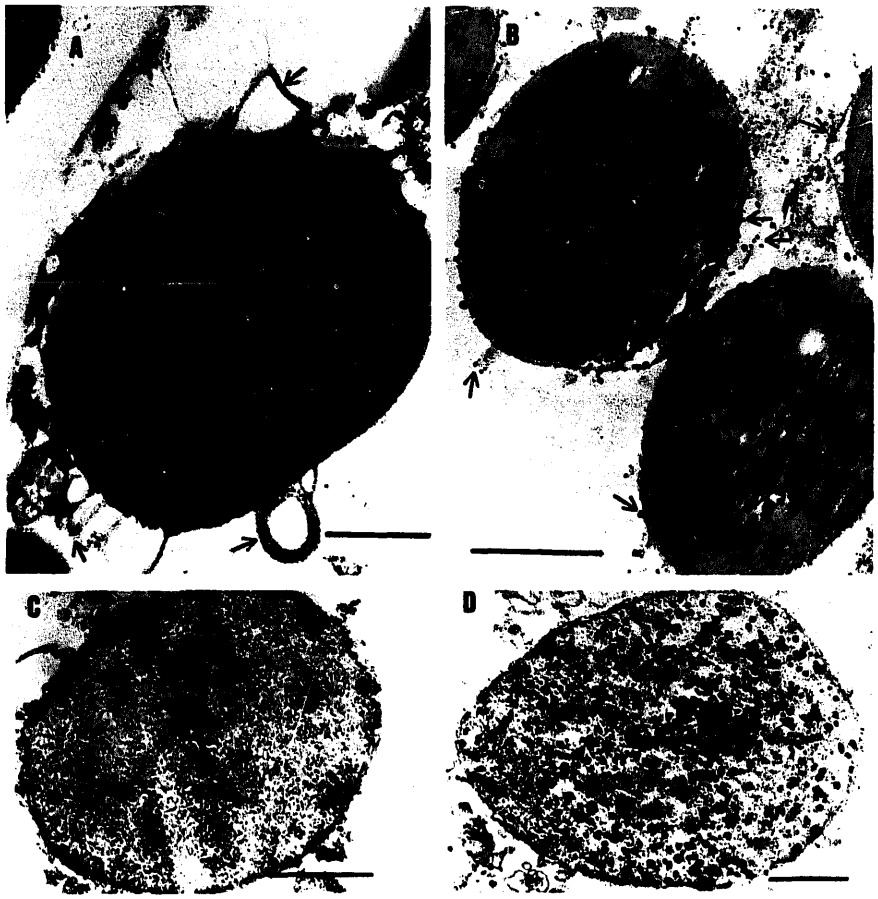

FIGURE 2. – Morphology of the 3 populations of lysosomal enzyme-containing structures. The fractions comprising the 3 populations of lysosomal enzyme-containing structures (see Figure 1) were separate by pooled and incubated in Gomori's medium to detect acid phosphatase. (a) Peak A, bottom of the gradient, density > 1.3; (b) peak B, density ≈ 1.22–1.25; (d) peak C, density ≈ 1.18; (c) peak C, control incubated in the absence of the substrate of acid phosphatase. *Arrows*: positive reaction. Bar = 1 μm.

ding to lysosomes, the activity banded at about 1.18 density, as described previously for *Artemia* lysosomes [13] and in general for other eukaryotes [3]. The activity at about 1.14 density probably originated from broken lysosomes.

Pattern of yolk degradation during Artemia *development*

Dormant embryos were incubated up to 60 hr and samples were taken every 2–4 hr. Larvae were synchronized immediately after hatching, thus the short incubation intervals were significant. For this purpose, dechorionization of the dormant embryos before incubation was important to improve synchrony.

Homogenates of embryos and larvae were centrifuged through sucrose isopycnic gradients as in Figure 1. Both acid phosphatase and cathepsin B activities were found to sediment in parallel in 3 peaks, although fewer than 3 peaks could be found at a given stage of development (Fig. 1). We called these peaks A, B, and C. Peak A, at the bottom of the gradient, has a density > 1.3; peak B, a density of 1.25–1.22, and peak C, a density of 1.19–1.17, as described above. The levels of enzyme activities found in the 3 different peaks during development are shown in Figure 4. The patterns of peaks A, B, and C shown in **a**, **b**, and **c** respectively, suggest an oscillatory pattern. The fact that the activity of the lysosomal enzymes is not constant during early development [13], probably explains that the peaks present different maxima. We reported previously [18] that intact yolk granules sediment to the bottom of the gradient (peak A). When the activity of peak A (acid phosphatase or cathepsin B) is represented as the percent of the total activity found at the corresponding time of development, the oscillatory pattern appears more distinct (Fig. 5). Lysosomal incorporation appears to take place mostly at about 0, 9, 17, 26, and 46 hr of incubation (Figs. 4**a** and 5), and the resulting degradative structures to accumulate progressively in peak B and peak C (Fig. 4). The enzymatic activity in peak B (Fig. 4**b**) reaches its maximum 5–15 hr after each period of

lysosomal enzyme incorporation in peak A. A further displacement in time of the maximum of peak C can also be observed (Fig. 4c). About 25% of the protein of peak A appears to have been degraded by hatching time and about 70% in the late larva (Fig. 5). These data probably indicate the fraction of yolk granules involved in the degradation process in the 2 developmental stages.

The relationship amoung peaks A, B, and C is more evident in Figure 6, where the densities of the maxima of the 3 peaks within each cycle are represented *versus* the time of development at which they were found. It can be seen how peak A becomes peak B and, in turn, peak C. By criteria of density, 5 cycles of yolk degradation seem to occur during development, as also suggested by criteria of activity in Figures 4 and 5.

DISCUSSION

The biochemical and morphological data presented suggest that the lysosomal system may be involved in the degradation of yolk granules in the crustacean *Artemia*. By sucrose isopycnic gradient centrifugation, 2 different lysosomal markers have been found to sediment in 3 peaks (Fig. 1). The buoyant density of 2 of the peaks (about $1.25 > 1.3$) is higher than that described for *Artemia* lysosomes (1.18, [13]), suggesting that during development, lysosomes are involved in the degradation of material of high density. The yolk granules of *Artemia* [18], have a high density (> 1.3) and therefore the finding of 2 heavy peaks suggested that they could be the resulting yolk degradative structures. The structures observed by electron microscopy revealed that the degradation was indeed to yolk granules and that acid phosphatase was associated with the resulting degradation structures (Fig. 2).

The data obtained are compatible with yolk degradation starting with the fusion of lysosomes to yolk granules. Yolk degradation appears to proceed with the clearance of the electron-dense material of the yolk granules (Fig. 2b). The induction of degradation has been observed *in vitro* upon incubation of yolk granules and lysosomes (Fig. 3). The degradation of yolk during *Artemia* development does not appear to be a continuous process (Figs. 5, 6). The lysosomal enzyme activities found associated with apparently intact yolk granules increased markedly at 5 specific times during the first 60 hr after resumption of development. Each increase has been interpreted as the initial step of 5 cycles of degradation of yolk granules.

The biochemical and morphological association of the lysosomal marker acid phosphatase with intermediates of yolk degradation supports our previous data suggesting the involvement of acid hydrolases in the degradation of yolk in *Artemia* [13]. We have also observed that yolk degradation can be inhibited by lysosomotrophic agents, that this inhibition leads to inhibition of development, and that the inhibition can be reversed upon removal of the drug [14]. In relation to the association of lysosomal enzymes to yolk granules, acid phosphatase has been used recently as the enzymatic marker of yolk platelets in sea urchin [1]. On the other hand, yolk proteins are long-lived and these kinds of proteins are thought to be degraded in the cell by the lysosomes through the formation of autophagolysosomes [10]. All these data may suggest that the involvement of the lysosomal system in yolk degradation is a more general phenomenon. The dependence of development on maternal resources suggests that the degradation of yolk should be a controlled process. We have suggested that as ATP is low at the beginning of development and is required for maintenance of the intralysosomal pH, it may be a factor involved in the control of yolk degradation [14].

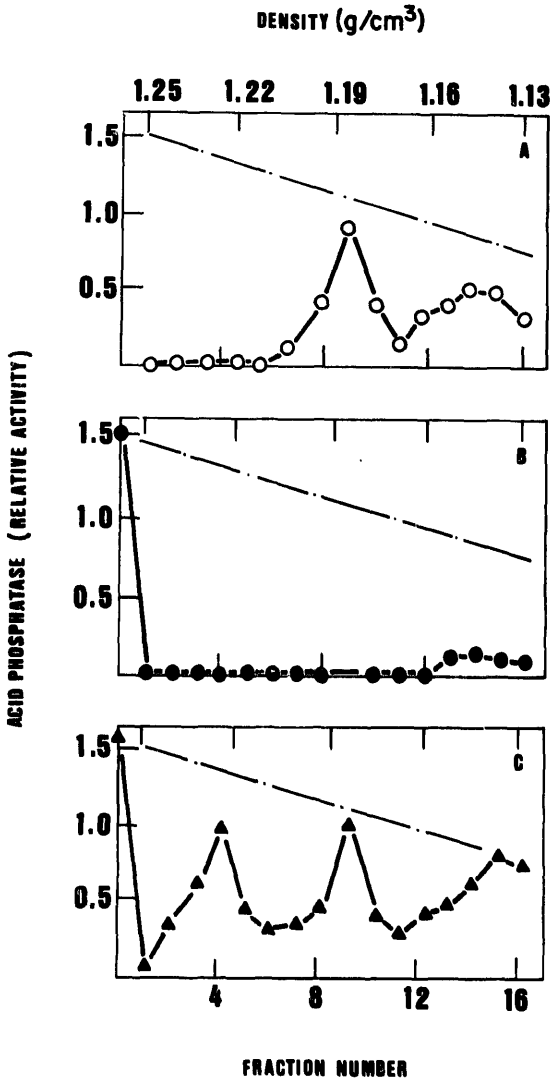

FIGURE 3. – Incubation of yolk granules and lysosomes induces the formation of a new peak of activity at density around 1.23. Yolk granules and lysosomes were obtained from dormant embryos as described ([18, 13] respectively). Samples of both subcellular fractions and their mixture (a, 0.85 ml of lysosome fraction; b, 0.35 ml of yolk granule fraction, and c, 1.2 ml of the mixture of both fractions) were run in parallel in sucrose gradients and analyzed as in Figure 1. The mixing of the 2 subcellular organelles induced the formation of a new peak of enzymatic activity at density around 1.23 (c) not present in the original component subcellular fractions (a, b).

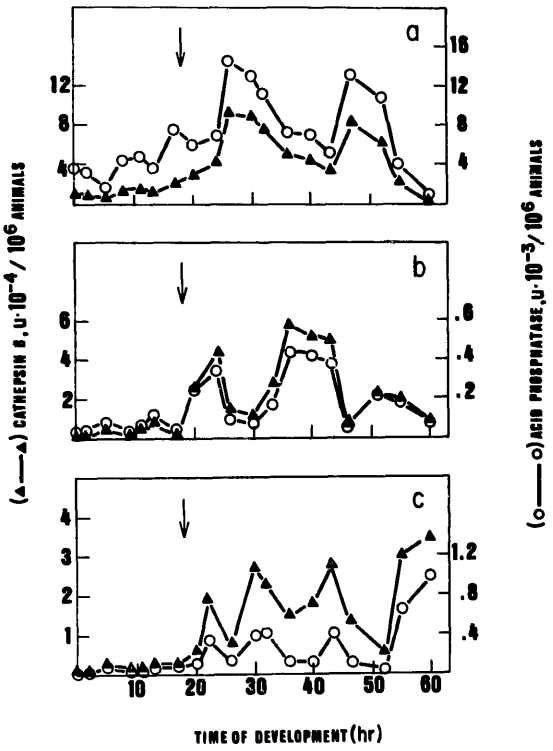

FIGURE 4. – Pattern of distribution of lysosomal enzyme activities among the 3 types of lysosomal structures during *Artemia* development. Homogenates (2 ml) of embryos and larvae at different stages of development were loaded on the sucrose isopycnic gradient, run, and analyzed as described for Figure 1. For each time of development tested, the total activity present in peak A, bottom of the gradient, density > 1.3 (a), in peak B, density ≈ 1.24 (b), and peak C, density ≈ 1.18 (c) is represented *versus* the time at which it was found. *Arrow*: hatching time.

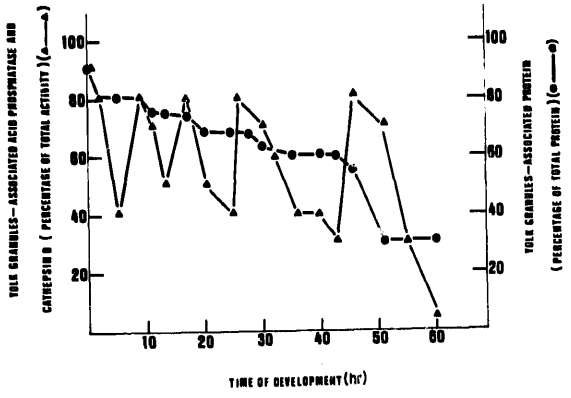

FIGURE 5. – Percentage associations of protein and lysosomal activity with yolk granules during development. The acid phosphatase and cathepsin B activities found in peak A (Fig. 4a) during development habe been calculated as the percentage of the total activity of the corresponding homogenates. The percentage data for the 2 activities (▲—▲) overlap. The protein of yolk granules in peak A in relation to the total protein of the corresponding homogenate has also been calculated (●—●).

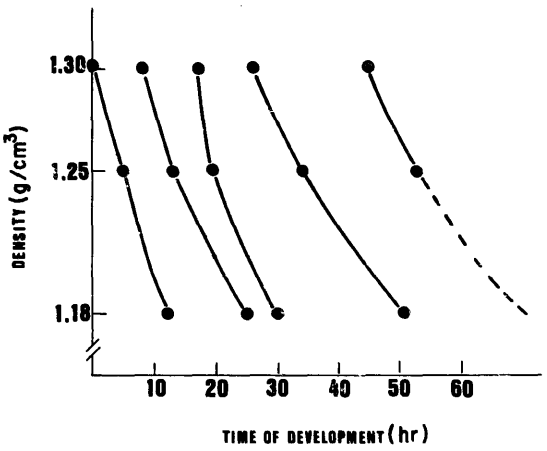

FIGURE 6. – The cycles of yolk degradation during *Artemia* development: temporal correlation of the buoyant density of the 3 populations of lysosomal structures. The buoyant density of the maxima of the 3 lysosomal structures (peaks A, B, C) was plotted *versus* the time of development at which each was reached. The points for each proposed degradation cycle are joined by a continous line.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Comisión Asesora de Investigación Científica y Técnica. R.P. had a fellowship from Caja de Ahorros de Madrid. We thank Drs. J. Renart and C.F. Heredia for critical reading of the manuscript. The assistance of A. Fernández with photography is acknowledged. We also thank E. Blanco for assistance with the electron microscope and Amador Ovejero with the figures.

NOTE

After this manuscript was submitted we became aware of the paper by Utterback and Hand (Am. J. Physiol., 252, 774–781, 1987). These authors have found that alkalinization of pH activates yolk granule disappearance in agreement with the activation of development observed before in these conditions. However, thay have not studied the involvement of proteases in the process. The only data given indicates that the serin–proteinase inhibitors PMSF and STI are note inhibitory in their conditions. Indeed, these inhibitors do not affect the activity of the cystein, cathepsin B-like proteinase of *Artemia* (Perona & Vallejo, 1989) that we suggest is involved in yolk protein degradation.

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